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Summary

The overall objective of this research is to examine the feasibility of accelerating wound healing by understanding the intra-cellular cellular mechanisms involved in the regulation of collagen synthesis. Collagen is a key component of all tissues. It is involved in defining the architecture and biomechanical characteristics of all tissue and organs and plays a major role in the modulation of cell proliferation, migration and differentiation. Collagen synthesis is a crucial step in wound healing.

The present studies are based on our hypothesis that the synthesis of collagen is regulated by the superoxide free radical (O_2°). Superoxide is produced in large amounts by phagocytes which infiltrate a wound in the early stages.

In the first two years of our study, our objective was to demonstrate a mechanistic relationship between (O_2°) and collagen synthesis and prepare the background for extending these studies to *in vivo* animal models in the continuation of the project. An *in vivo* wound chamber model using rabbits was adapted from our collaborative studies with colleagues at UCSF and used for initial studies on wound healing. As the Contract ended at the end of the initial two years, we have reportable data only for the effect of (O_2°) on collagen synthesis in the *in vitro* tissue culture model for wound healing.

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Our studies showed that IMR-90 fibroblasts derived from human fetal lung tissue are a suitable model for investigations on collagen synthesis. These cells retain many of the biochemical characteristics of fibroblasts in tissues. We examined the synthesis of collagen and the levels of the collagen related enzyme prolyl hydroxylase in these cultures in the presence or absence of (O_2^0). Exposure to (O_2^0) resulted in a marked stimulation of collagen synthesis and an increase in the activity of prolyl hydroxylase. We also examined the nature of collagen synthesized in the presence and absence of (O_2^0). These experiments showed that (O_2^0) increased the synthesis of type III collagen to a much greater extent than the synthesis of type I collagen. This observation is consistent with the biochemical events occurring in repairing wounds *in vivo*. Thus our studies supported our hypothesis that an increase in (O_2^0) levels increases the synthesis of collagen and suggest that measures to increase (O_2^0) may also promote collagen synthesis in healing tissues *in vivo* and facilitate wound healing.

Introduction

The role of oxygen wound healing is well recognized and documented in our original proposal. The availability of oxygen is critical for repair processes. The beneficial effects of hyperbaric oxygen therapy on wound repair have been amply demonstrated. Increased oxygen supply promotes wound healing whereas decreased oxygen supply retards repair. The beneficial effects of oxygen on wound healing can be directly correlated with its direct involvement in collagen synthesis which is crucial to repair. Collagen is responsible not only for tissue architecture and biomechanical characteristics, but also plays a regulatory role as it modulates cell proliferation, migration and anchorage, and contributes to cell and tissue differentiation. Oxygen is a substrate for the extremely important post-translational hydroxylation of proline and lysine residues in collagen.

Many lines of evidence suggest that oxygen may also act at the genetic and epigenetic levels to modulate the expression of collagen genes and the expression of genes for the enzymatic machinery required in the post-translational modification of collagen. The active species in this regulatory role of oxygen is not the oxygen molecule but a product of its metabolism, superoxide free radical (O_2°). Superoxide is the product of univalent reduction of oxygen, the first step in the biological fixation of oxygen. The (O_2°) free radical is a highly reactive species which can react with a variety of important molecules and macromolecules in the cell, however, it is well known

that its interaction with the genetic material of cells results in a train of events that results in the unmasking and increased expression of certain genes. Our hypothesis is that the (O₂[°]) free radical is a major stimulant for collagen synthesis and that it should be possible to improve the efficiency of wound repair by increasing the tissue levels of (O₂[°]) free radical. In fact during normal wound healing, an important event is the generation of (O₂[°]) free radical by phagocytic cells of the immune response which sets the pace of repair. It is our belief that an understanding of the regulation of collagen synthesis by superoxide can lead to the development of procedures for promoting wound healing, especially under those circumstances where oxygen delivery may be impaired by hypervolemia and other trauma related factors. In these studies we have examined the participation of (O₂[°]) free radical in collagen synthesis to confirm its involvement in the regulation of collagen synthesis and to elucidate the mechanisms involved in the modulation of collagen synthesis. Our studies are summarized in the article reproduced in the following pages.

STIMULATION OF COLLAGEN SYNTHESIS IN FIBROBLAST CULTURES BY SUPEROXIDE

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Abstract: Exposure of diploid fetal human fibroblasts (IMR-90) to superoxide generated by dihydroxyfumarate resulted in increased collagen synthesis. The synthesis of type III collagen was stimulated to a greater extent than the synthesis of type I collagen. The stimulation of collagen synthesis was abolished by superoxide dismutase. Our observations suggest that superoxide may play a role in the regulation of collagen synthesis and may modulate differential collagen gene expression. These observations may explain the increased synthesis of collagen in tissues following inflammation or exposure to oxidant conditions.

Key words: Superoxide, stimulation, collagen synthesis, type I collagen, type III collagen, fibroblasts

STIMULATION DE LA SYNTHÈSE DU COLLAGÈNE EN CULTURES DE FIBROBLASTES PAR LE SUPEROKYDE

Résumé: L'exposition de fibroblastes diploïdes fœtaux humains (IMR-90) à du superoxyde générée par le dihydroxyfumarate a entraîné une augmentation de la synthèse du collagène. La synthèse de collagène type III a été stimulée à un degré plus fort que celle du collagène type I. La stimulation de la synthèse de collagène a été abolie par la superoxyde dismutase. Nos observations suggèrent que le superoxyde joue un rôle dans la régulation de la synthèse de collagène et puise moduler une expression différentielle des gènes du collagène. Ces observations peuvent expliquer l'augmentation de la synthèse de collagène dans les tissus, suite à une inflammation ou à une exposition à des conditions oxydantes.

Mots clés: Superoxide, stimulation, synthèse du collagène, collagène type I, collagène type III, fibroblastes

INTRODUCTION

Several lines of evidence suggest a role for oxygen and its metabolites in the regulation of collagen synthesis. Inflammation, a pathobiological condition characterized by the generation of superoxide free radical O_2^- , leads to excess collagen synthesis and deposition in tissues (Barnes *et al.*, 1976; Kent *et al.*, 1976). The synthesis of collagen during wound repair is modulated by the availability of oxygen (Knighton *et al.*, 1982). Exposure of tissues to hyperoxic conditions (Chvapil and Peng, 1975) and oxidants such as ozone (Bhatnagar *et al.*, 1983) results in increased collagen synthesis. In addition, agents such as Bleomycin and paraquat (methyl viologen) which generate O_2^- also stimulate collagen synthesis (Hussain and Bhatnagar, 1979; Hussain *et al.*, 1985). Because of the importance of inflammation in fibrotic disease, it is of interest to examine the mechanisms involved in the stimulation of collagen synthesis under these conditions. We have investigated the synthesis of collagen in the presence of exogenous O_2^- in IMR-90 diploid fibroblasts.

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MATERIALS AND METHODS

Cell Culture Procedures

Diploid fibroblasts (IMR-90) derived from fetal human lung were obtained in the fifth and sixth passages from the Institute for Medical Research, Camden, NJ. All experiments were carried out in the eighth to twelfth passages. The cells were grown to confluence in 60 mm dishes (Falcon Plastics) in Dulbecco's modification of Eagles minimum essential medium supplemented with 10% fetal bovine serum.

Exposure of Fibroblasts to Superoxide

The cells were exposed to O_2 in the presence of dihydroxyfumarate (DHF), a well known generator of the free radical (Fischer, 1981). Previous studies (AATAR *et al.*, 1980) showed that DHF generates O_2 in tissue culture media and that the free radical produced under these conditions is scavenged by superoxide dismutase. We used this agent because its other metabolites are not known to have significant effects on cells, and therefore the observed cellular effects can be attributed to the production of oxygen metabolites in the culture medium.

Preliminary experiments using Trypan Blue exclusion assay showed no cytotoxic effects in the presence of DHF in concentrations used in these experiments. The lack of significant cytotoxicity at DHF concentrations lower than $60\mu M$ was confirmed by experiments in which the incorporation of radiolabelled precursors into cellular macromolecules was examined.

Collagen Biosynthesis

Culture proteins were labelled with $10\mu Ci$ [$U^{14}C$] - proline (268 mCi/mMole, New England Nuclear) for 24 hrs., in the presence of 3-aminopropionitrile, an inhibitor of collagen crosslinking (Prockop and Tuderman, 1982) to facilitate the analysis of collagen. This agent is not known to affect O_2 or SOD. For analysis of radioactive incorporation and collagen synthesis, the medium and cell layer were harvested and dialyzed against H_2O in the presence of 1 mM ethylene diamine tetraacetic acid and 1 mM phenyl methyl sulfonyl fluoride at 4° . Control experiments showed over 95% recovery of labelled protein by this procedure. The dialyzed samples were hydrolyzed in 6 N HCl for 24 hrs. at 110° and the hydrolysates were analyzed for [^{14}C]-hydroxyproline by published methods (Juva and Prockop, 1966). DNA content as a measure of cell population was determined by the diphenylamine method (Bunton, 1956). Total protein content was determined by Coomassie Brilliant Blue procedure (Bradford, 1976).

Analysis for Type I and Type III Collagen

In order to isolate and quantitate the newly synthesized type I and type III collagens, both the conditioned medium and the cell layer were subjected to limited pepsin digestion to facilitate the complete solubilization and chain separation of the collagens. The collagens were separated by fractional salt precipitation with 1.7 M and 2.6 M NaCl at pH 7.0 (Miller and Rhoads, 1982). The labelled collagen chains were separated by chromatography on columns of carboxymethyl cellulose. The identity of the chains was confirmed by polyacrylamide gel electrophoresis.

RESULTS

In preliminary experiments (data not shown), we determined the optimal concentration of DHF for use in these studies. Since hydroxyproline synthesis occurs as a result of intracellular hydroxylation of proline incorporated in polypeptide precursors of collagen, we followed the synthesis of [^{14}C]-hydroxyproline as a measure of collagen synthesis. The amount of radioactivity incorporated into hydroxyproline measured as a function of DNA content increased in the presence of DHF. Maximal increase in hydroxyproline synthesis was achieved at DHF concentrations of 10-20 μM and no further increase occurred beyond these concentrations. All subsequent experiments were carried out at DHF concentrations of 10 μM . Maximal collagen synthesis in fibroblast cultures occurs when the cultures become confluent (Peterkofsky, 1972). All experiments reported here were carried out with confluent cultures. In order to examine the possibility that the effect of DHF on collagen synthesis may have resulted from an increase in the number of cells, we determined the total DNA and protein content of the cultures (Fig. 1). Exposure of cells to O_2 for periods upto 24 hrs. did not result in any increases in DNA or protein content, indicating that the free radical did not induce cell proliferation in the confluent cultures. The increase in [^{14}C] hydroxyproline synthesis as a function of the DNA content of the cultures was apparent as early as 6 hrs. after the addition of DHF (Fig. 2). The increase was linear during the 24 hrs. period of observation.

In order to confirm if the increase in collagen synthesis in the presence of DHF resulted from generation of O_2 , the effect of removing the free radical by superoxide dismutase was examined. As seen in Table 1, exposure of cultures to 10 μM DHF for 24 hrs. resulted in a 23% increase in the incorporation of [^{14}C]-proline and a 130%

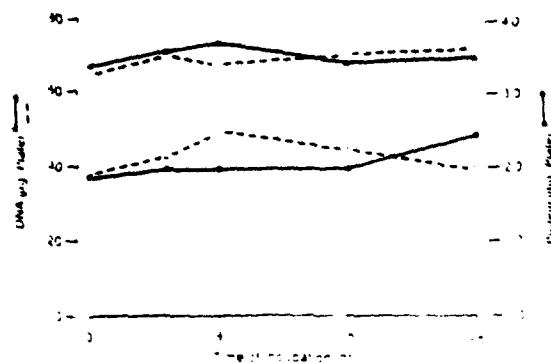


Fig. 1

Fig. 1 *The lack of effect of DHF on cell growth:* Confluent cultures were exposed to 10 μ M DHF for 24 hrs. and DNA and protein content were determined as described. Each value is the mean of triplicate cultures. Open circles and triangles represent data from control cultures, filled circles and triangles represent DHF-treated cultures.

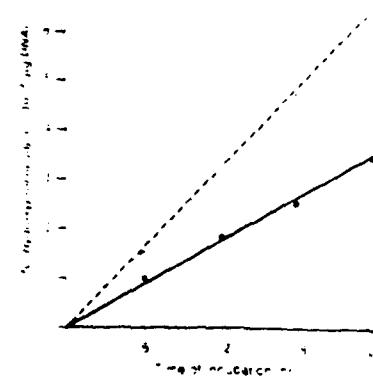


Fig. 2

Fig. 2 *Increased collagen synthesis in the presence of DHF:* Confluent cultures were exposed to 10 μ M DHF and labelled with [14 C]-proline for various times. The content of [14 C]-hydroxyproline was determined as described in Methods. Each point is the average of triplicate cultures. Closed circles represent control, and open circles represent data from DHF-treated cultures.

TABLE I $[^{14}\text{C}]$ -Proline Incorporation and Synthesis of $[^{14}\text{C}]$ -hydroxyproline in the presence of DHF

Radioactivity Incorporated (dpm/ μ g DNA $\times 10^{-4}$)			
	Treatment	Total Radioactivity	$[^{14}\text{C}]$ -hydroxyproline
Experiment 1	Control	188.7 \pm 21	3.39 \pm 0.33
	+ DHF, 10 μ g/ml	231.5 \pm 22 (+23%)*	7.81 \pm 0.77 (+130%)**
	+ DHF, 10 μ g/ml + SOD	190.0 \pm 18	3.83 \pm 0.39
Experiment 2	Control	159.6 \pm 18	2.76 \pm 0.30
	+ DHF, 10 μ g/ml	211.9 \pm 23 (+32%)*	8.03 \pm 0.85 (+196%)**
	+ DHF, 10 μ g/ml + SOD	161.2 \pm 17	2.81 \pm 0.30

Confluent cultures were incubated for 24 hrs. in the presence of DHF and [14 C]-proline with or without SOD. In experiments with SOD, the cells were equilibrated in SOD-containing medium for 30 min. before the addition of DHF. The numbers in parentheses indicate percent change from control. Each number is the mean \pm S.D. of five determinations. * $P < 0.05$, ** $P < 0.001$.

increase in the synthesis of [14 C]-hydroxyproline, on a per DNA basis. Since hydroxyproline is present in significant amounts only in collagen, these data suggest that the increased incorporation of proline could be accounted for largely by increased collagen synthesis. This increase was abolished by superoxide dismutase added at a concentration of 10 U/ml in the culture medium. Heat denatured superoxide dismutase had no effect. These data support the concept that the stimulatory effect of DHF on collagen synthesis may be mediated by O_2^- .

The expression of collagen phenotypes is altered in tissues under conditions characterized by increased exposure to O_2^- (Bailey *et al.*, 1972; Baseman *et al.*, 1981; Clove *et al.*, 1979). We examined the nature of collagen synthesized

in the presence of DHF. The labelled procollagens were subjected to limited pepsin digestion to ensure complete recovery from the matrix, and were differentially precipitated with NaCl to separate type I and type III chains. The chains were further purified by chromatography on carboxymethyl cellulose columns and the radioactivity incorporated in type I and type III chains was measured. These data are presented in Table 2. As seen in Table 2, i.e. synthesis of type III collagen was increased by 42% whereas there was no significant increase in type I collagen synthesis.

TABLE 2 *Relative Synthesis of Type I and Type III Collagens in the Presence of DHF*

Treatment	*Radioactivity, dpm x 10 ⁻⁴		Type III/Type I
	Type I	Type III	
Control	1.82 ± 2.3	0.32 ± 0.35	0.176
+DHF, 10 µg/ml	1.91 ± 2.1	0.46 ± 0.41 (+42%)**	0.238

*Radioactivity in pooled fractions from carboxymethyl cellulose chromatography of differential salt precipitates of type I and type III collagens. Each value is the average of triplicate experiments ± S.D. The number in parenthesis denotes percent change from control. ** P < 0.05.

DISCUSSION

Our studies suggest that O₂ may directly stimulate the synthesis of collagen. Other studies in our laboratory showed that the free radical induces increased prolyl hydroxylase activity in IMR-90 fibroblasts. The increase in prolyl hydroxylase activity in the presence of O₂ was abolished by scavengers of the free radicals. These data provide an explanation for the increase in collagen synthesis observed in tissues under conditions that elevate O₂ levels, including inflammation (Babior, 1982), exposure to increased oxygen (Chvapil and Peng, 1975), oxidants such as ozone (Bhatnagar *et al.*, 1983), and exposure to O₂ generating agents such as paraquat and Bleomycin (Hussain *et al.*, 1983; Hussain and Bhatnagar, 1979). The observation that anti-inflammatory agents decrease the production of O₂ (Oyangui, 1976) and cause a lowering of collagen synthesis (Kruse *et al.*, 1978; Phan *et al.*, 1981) also supports this concept.

Type III collagen synthesis is markedly enhanced in tissues in early stages of fibrosis and other scarring processes (Bateman *et al.*, 1981; Kent *et al.*, 1976; Kruse *et al.*, 1978; Phan *et al.*, 1981). Type III collagen also accumulates in repair tissue during wound healing (Bailey *et al.*, 1972; Clove *et al.*, 1979). The greater increase in type III collagen synthesis in the presence of O₂ observed by us suggests that the free radical may play a role in the modulation of differential collagen gene expression.

The present studies do not provide information as to the mechanisms of regulation of collagen synthesis by O₂. The free radical is known to affect cell growth and differentiation (Michelson, 1982; Michelson and Buckingham, 1974). The genetic level regulatory effects of the free radical may arise from its interaction with informational macromolecules (Brown and Fridovich, 1981; Leska *et al.*, 1980; Moody and Hassan, 1982). Interaction of the free radical with specific sites on the chromatin may result in the expression of the genetic complex for collagen synthesis, including the enzymes involved in its post-translational processing. In higher organisms the inflammatory response elicited by foci of irritation such as injury, bacteria, or particulate material elicits the synthesis of collagen to wall off the irritant stimulus. Collagen plays a protective role by forming a tough and impenetrable barrier against injurious stimuli including radiation and oxidants. It has been suggested that (Cerruti, 1985) tissue pro-oxidant states characterized by increased levels of oxygen-derived free radicals may induce the expression of certain genes that are a part of the organism's defensive response, reminiscent of the SOS function in bacteria. It is interesting to speculate that teleologically collagen and its biosynthetic machinery may be a part of such pro-oxidant mechanisms in animal cells.

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Conclusions

Our studies support the hypothesis that superoxide is a stimulant for collagen synthesis. It promotes the overall synthesis of collagen, but the increase in type III collagen synthesis is much greater than that observed in type I collagen synthesis. Type III collagen is a fetal type collagen that is involved in development and differentiation of tissues. Increasing levels of type III collagen in wound tissues can be expected to have beneficial effects on tissue repair and regeneration. Our studies support the concept that increased delivery of superoxide to wounds may accelerate and facilitate repair. We suggest continuing studies along these lines to

- Examine the effect of superoxide on collagen synthesis *in vivo*,
- Examine the effect of increased superoxide levels on wound healing, and
- Develop strategies to administer superoxide to wounds.